NLRP3 (NALP3, Cryopyrin) Facilitates In Vivo Caspase-1 Activation, Necrosis, and HMGB1 Release via Inflammasome-Dependent and -Independent Pathways

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Stephen B. Willingham,2* Irving C. Allen,2† Daniel T. Bergstralh,2† Willie June Brickey,‡ Max Tze-Han Huang,§ Debra J. Taxman,§ Joseph A. Duncan,¶ and Jenny P.-Y. Ting3*§

Bacterial infection elicits a range of beneficial as well as detrimental host inflammatory responses. Key among these responses are macrophage/monocyte necrosis, release of the proinflammatory factor high-mobility group box 1 protein (HMGB1), and induction of the cytokine IL-1. Although the control of IL-1β has been well studied, processes that control macrophage cell death and HMGB1 release in animals are poorly understood. This study uses Klebsiella pneumoniae as a model organism because it elicits all three responses in vivo. The regulation of these responses is studied in the context of the inflammasome components NLRP3 and ASC, which are important for caspase-1 activation and IL-1β release. Using a pulmonary infection model that reflects human infection, we show that K. pneumoniae-induced mouse macrophage necrosis, HMGB1, and IL-1β release are dependent on NLRP3 and ASC. K. pneumoniae infection of mice lacking Nlrp3 results in decreased lung inflammation and reduced survival relative to control, indicating the overall protective role of this gene. Macrophage/monocyte necrosis and HMGB1 release are controlled independently of caspase-1, suggesting that the former two responses are separable from inflammasome-associated functions. These results provide critical in vivo validation that the physiologic role of NLRP3 and ASC is not limited to inflammasome formation.


The NLR (nucleotide binding domain, leucine rich repeats-containing) family (formerly known as CATERPILLER, NOD, NACHT-LRR, and NOD-like receptor) family of genes/proteins is increasingly implicated in the regulation of immunity (1). The NLR family member NLRP3 (formerly cryopyrin, CIAS1, and NALP3), which is expressed abundantly in neutrophils and macrophages, has emerged as a critical mediator of inflammation. NLRP3/CIAS1, the human gene encoding this protein, was first identified through its association with the hereditary periodic fever syndrome CAPS (CIAS1-associated periodic syndrome), which comprises a wide range of inflammatory symptoms and severity (2–6). This condition results from gain-of-function mutations in NLRP3. Disease-associated variant forms of NLRP3 exhibit enhanced capacity to induce caspase-1 and IL-1β maturation and a necrotic pathway of macrophage cell death that indirectly enhances inflammation (7–9).

A role for NLRP3 in caspase-1 maturation is well studied. Following stimulation, NLRP3, the adaptor ASC (apoptotic speck-like protein containing a caspase recruitment domain), Cardinal/TUCAN, and procaspase-1 combine to form one of several known inflammasome complexes. Within this complex, procaspase-1 is activated that in turn cleaves and activates the pyrogenic cytokines IL-1β and IL-18 (9). Currently, the inflammasome protein complex has also been demonstrated for NLRP1 (NALP1), and the function of caspase-1/IL-1β release has been associated with NLRC4 (IPAF/CLAN) and NAIP5; however, activation of the NLRP3 inflammasome is associated with the widest spectrum of stimuli. Among these are Gram-positive and Gram-negative bacteria, including Staphylococcus aureus, Listeria monocytogenes, and Shigella flexneri, toxins as well as uric acid crystals, and pathogen- or nonpathogen-derived nucleic acids (8, 10, 11). Environmental pollutants such as asbestos and silica, as well as particulate adjuvants and β-amylloid, have also been found to require NLRP3 for caspase-1-mediated cytokine secretion (12–16).

Recent work has suggested a second proinflammatory function for NLRP3 involving monocyte necrosis (7, 8). Although the study of this process has been previously limited to in vitro culture, this form of necrosis has been shown to occur in monocytes cells infected with intracellular bacteria or following exposure to toxins. Both microbial pathogen (S. flexneri)-induced necrosis and necrotic death associated with CAPS require NLRP3, its partner protein ASC, and the lysosomal protease cathepsin B (8). However, NLRP3-dependent necrosis is entirely independent of caspase-1 and IL-1β and other apoptosis-associated caspases and has been named pyro necrosis. One defining feature of monocyotic necrosis is the loss of plasma membrane integrity and the subsequent spilling of intracellular inflammatory contents, most notably the nuclear
factor high-mobility group box protein 1 (HMGB1),\(^4\) which elicits strong proinflammatory effects when released into the microenvironment (17). HMGB1 can activate RAGE (receptor for advanced glycation end products) and TLRs to elicit proinflammatory responses, including the release of TNF-\(\alpha\) and IL-1\(\beta\). HMGB1 is considered a therapeutic target, as Abs against HMGB1 have been shown to effectively reduce sepsis, arthritis, and cancer in animals (18–20). However NLR-dependent inflammatory necrosis or HMGB1 release remains to be validated in a physiologic setting (21).

*Klebsiella pneumoniae* is among the most common Gram-negative bacteria encountered by clinicians worldwide and is a leading cause of community-acquired and hospital-associated respiratory infection (22). Its frequency in the latter context is particularly alarming, as *K. pneumoniae* is responsible for up to 23% of nosocomial infections and a mortality rate of up 50% in elderly or otherwise compromised patients (23). Moreover, the growing prevalence of antibiotic-resistant strains in this species has led to increased attention and concern (24, 25). *K. pneumoniae* is a non-motile, nonflagellated, Gram-negative, rod-shaped bacterium that normally resides within the mouth, skin, and intestines. Pathogenic *K. pneumoniae* invades the lungs where it is capable of inducing severe bacterial pneumonia that is often complicated with bacteremia and sepsis (26). Airway infection typically leads to extensive lung injury resulting from increased inflammation, hemorrhage, and the necrotic destruction of lung tissue. This process results in thick, blood-laced mucous known as “currant jelly” sputum, which is characteristic of *K. pneumoniae*-induced pneumonia. In addition to cellular necrosis, this bacterium also induces HMGB1 release in humans (27). Although recent work has started to identify immune mechanisms underlying these diverse host responses to bacterial infection, little work has been done to examine the regulation of the diverse inflammatory responses elicited by *K. pneumoniae* infection or the contribution of these mechanisms to pathogenesis or immunity (28–30). Due to the multiple inflammatory responses that are elicited by *K. pneumoniae*, including cytokines, necrosis, and HMGB1 induction, and its potential as a public health threat caused by the rise of antibiotic-resistant strains, we considered a therapeutic target, as Abs against HMGB1 have been increased attention and concern (24, 25).

### Experimental animals

All studies were conducted in accordance with National Institutes of Health guidelines for the care and use of laboratory animals and approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill. Mice deficient in *Nlrp3, ASC, Nlr4*, and the caspase-1 gene were generated as previously described (31, 32). All animals were maintained in pathogen-free facilities at the University of North Carolina at Chapel Hill.

### Materials and Methods

#### Experimental animals

*K. pneumoniae* 43816, serotype 2, was obtained from the ATCC and cultured inuria-Bertani broth. Bacterial density was estimated by measuring the absorbance at 600 nm (1 OD\(\text{nm}=3\times10^{8}\) bacteria/ml). Accurate CFU were determined for each experiment by plating an aliquot on Luria-Bertani agar plates.

#### Bacteria-induced inflammation

Cultures of *K. pneumoniae* were pelleted, washed twice in PBS, and resuspended in PBS. Mice were anesthetized and challenged via intratracheal instillation with 7.4 \(\times\) 10\(^7\) CFU of *K. pneumoniae* in 50 \(\mu\)l of PBS as previously described (28). Mock-challenged mice received 50 \(\mu\)l of PBS. THP-1 cells were infected with *Klebsiella at a multiplicity of infection* (MOI) of 50 for 6 h. Bone marrow-derived macrophages (BMDM) were prepared as previously described (8) and infected with *Klebsiella* (MOI of 200 for 6 h) or *Salmonella* (MOI of 50 for 1 h). Samples were centrifuged at 650 \(\times\) g for 10 min immediately following the addition of bacteria. Gentamicin (50 \(\mu\)g/ml) was added to cultures 1 h postinfection.

#### Assessment of bacterial burden

Mice were euthanized via i.p. injection with 2,2,2-tribromoethanol (Avertin). Whole liver, spleen, and lungs were removed, their wet weight was assessed, and they were homogenized in 500 \(\mu\)l of HBSS with a Tissue Master 125 (Omni International) and centrifuged. The resulting supernatants were plated on Luria-Bertani agar plates.

#### Assessments of airway inflammation

Mice were euthanized via i.p. injection of Avertin and serum was harvested via cardiac puncture. The liver, kidney, and spleen were removed, weighed, and either homogenized in 500 \(\mu\)l of HBSS; and shNLRP3, 5'-GCTCTTCCTGGCCACACCA-3'; and shLRP3, 5'-GGATGAAACCTGTTCACACCA-3'.

#### Histopathologic examination

For histopathological examination, lungs were fixed by inflation (20-cm pressure) or fixed in 4% paraformaldehyde. Mice were then perfused with HBSS followed by centrifugation to isolate bronchoalveolar lavage cells and cell-free supernatants. RBC were lysed via hypotonic saline treatment and cellularity was assessed with a hemacytometer. Aliquots of bronchoalveolar lavage fluid (BALF) were cytopsin onto slides and stained with Diff-Quik (Dade Behring) for differential cell counts. Leukocytes were identified based on the morphology of \(>200\) cells per sample. An aliquot of BALF was plated on Luria-Bertani agar to assess bacterial burden. The remaining BALF was centrifuged and the supernatant was collected. Following BALF harvest, the lungs were fixed by inflation (20-cm pressure) and immersed in 4% paraformaldehyde.

### Bacteria

*K. pneumoniae* 43816, serotype 2, was obtained from the ATCC and cultured inuria-Bertani broth. Bacterial density was estimated by measuring the absorbance at 600 nm (1 OD\(\text{nm}=3\times10^{8}\) bacteria/ml). Accurate CFU were determined for each experiment by plating an aliquot on Luria-Bertani agar plates.
Cytokine and chemokine assessment

Cell-free supernatants from *K. pneumoniae* (MOI of 200 for 6 h) or *Salmonella typhi*-infected (MOI of 50 for 1 h), thiglycolate-elicted peritoneal macrophages were analyzed using RayBiotech cytokine Ab array G, series 3 (RayBiotech). An Axon scanner 4000B with GenePix software was used to collect fluorescence intensities. These values were normalized to the ratio of positive control values for each sample. Total normalized fluorescence values of replicate spots were averaged and expressed as fold increase over noninfected samples. N/D, for “not determined,” indicates cytokines for which the fluorescence values of replicate spots deviated ≥2-fold and were thus dismissed. If this occurred in the nontreated sample, the cytokine was removed from the data set. Cytokine concentrations were determined by RayBiotech using its Quantibody service.

**ELISA**

Samples were collected at the indicated times and assayed with OptEIA human IL-1β ELISA set or OptEIA mouse IL-1β or IL-6 ELISA sets (BD Biosciences).

**Cell viability determination**

Viability was assayed per manufacturer protocol using either CytoTox-ONE homogeneous membrane integrity assay (Promega), ToxiLight Biosay kit (Lonza Biosciences), or 7-aminoactinomycin (7-AAD; BD Biosciences) staining as indicated. In the case of 7-AAD staining, cells were collected, washed twice in PBS, resuspended in 0.5 ml PBS with 1 μl 7-AAD, and incubated for 15 min before analysis on a FACSscan flow cytometer (BD Biosciences).

**Results**

*K. pneumoniae*-induced cell death and IL-1β release require NLRP3 and ASC

The role of NLR proteins in the host immune response to *K. pneumoniae* was first examined in a human macrophage cell line. We observed both IL-1β release (Fig. 1A) and cell death (Fig. 1B) following the infection of THP-1 human monocytic cells with *K. pneumoniae*. Both processes were abrogated in a human monocytic cell line with reduced NLRP3 expression (Fig. 1, A and B). Reduction of NLRP3 was achieved through the stable integration of retroviruses encoding shRNA designed to promote the targeted degradation of NLRP3 mRNA (labeled shNLRP3), as described and demonstrated previously (8, 34).

Previously, we demonstrated that ASC, a partner protein of NLRP3, is required for *S. flexneri*-induced cell death in THP-1 cells (8). Therefore, we tested the ability of Klebsiella to elicit cell death and IL-1β in ASC-deficient THP-1 cells. Both IL-1β release and cell death were substantially abrogated in the shASC cells (Fig. 1, C and D). Having established that NLRP3 is essential for Klebsiella-induced cell death, we sought to determine the nature of this phenomenon. Indicative of pyrocnescence, *K. pneumoniae*-induced cell death was markedly reduced in control THP-1 cells treated with the cathepsin-B inhibitor Ca-074-Me (Fig. 1E). The caspase-1 specific inhibitor YVAD-CHO had no effect on cell death as measured by lactate dehydrogenase (LDH) release (Fig. 1E). The caspase-1-specific inhibitor was used at concentrations sufficient to inhibit caspase activity, as evidenced by the attenuation of Klebsiella-induced IL-1β release (Fig. 1F). Interestingly, the cathepsin B inhibitor not only blocked Klebsiella-induced cell death in THP-1 cells but also prevented IL-1β release in response to the pathogen (Fig. 1F). This indicates that cathepsin B also controls IL-1β release.

Additional features of Klebsiella-induced cell death are also consistent with pyrocnescence. During apoptosis, caspase-3 undergoes activating cleavage. In turn, caspase-3 cleaves PARP and other downstream substrates. Neither caspase-3 nor PARP were cleaved in shCTRL or shNLRP3 cells infected with Klebsiella, although both were cleaved in staurosporine-treated cells (Fig. 1G). During macrophage necrosis, HMGB1 is released. In accordance with this, HMGB1 is released from *K. pneumoniae*-infected shCTRL cells but not from infected shNLRP3 cells as determined by Western blotting (Fig. 1H). HMGB1 is not released by THP-1

![FIGURE 1. Klebsiella pneumoniae-induced cell death and IL-1β release](http://www.jimmunol.org/Downloaded)
cells treated with staurosporine, a well-established inducer of apoptotic cell death (Fig. 1H). HMGB1 release from shCTRL cells is abrogated by a caspase B inhibitor (Ca-074-Me), indicating that Klebsiella-induced HMGB1 release requires cell death (Fig. 1I).

**Klebsiella-induced IL-1β is reduced in Nlrp3−/− and Asc−/− macrophages**

To examine the physiologic importance of these results, BMDM were isolated from wild-type (WT) mice and mice deficient for Nlrp3, Asc, or Nlrc4. Deletion of Nlrp3 or Asc resulted in a near complete inhibition of IL-1β induced by Klebsiella as measured by ELISA, whereas Nlrc4-null macrophages demonstrated no substantial difference from WT (Fig. 2A). Importantly, this phenomenon is not common to all pathogenic bacteria. In agreement with previous work, IL-1β release from macrophages infected with S. typhi was unaffected by Nlrp3 deletion, whereas deletion of Nlrc4 eliminated the inflammatory response (Fig. 2A). The NLRP3 inflammasome was previously reported to be activated by a combination of Escherichia coli LPS and ATP (9). To determine whether the NLRP3 inflammasome is also activated by Klebsiella LPS, BMDM were challenged with 50 ng/ml LPS isolated from K. pneumoniae for 16 h in combination with ATP (5 mM for 20 min), stimulated NLRP3 and ASC-dependent but NLRC4-independent IL-1β release from BMDM. Addition of either 100 μM caspase-1 inhibitor (YVAD) or 50 μM caspase B inhibitor (Ca-074-Me) substantially abrogated K. pneumoniae induced IL-1β release from BMDM. IL-1β was measured by ELISA. NT, No treatment; N/D, not detected.

**K. pneumoniae induces chemotactic and inflammatory cytokine production in primary mouse macrophages**

The processing and release of proinflammatory cytokines and chemokines is fundamental to proper innate immune response to pathogens. To more broadly assess the effect of NLRP3 and ASC on host inflammatory responses, cell-free supernatants prepared from Klebsiella- or Salmonella-infected macrophages were analyzed on anti-cytokine Ab arrays. Cytokines and chemokines induced ≥5-fold over the nontreated control of each genotype are shown. B, Quantification of IL-1β, MCP-1, and KC (keratinocyte-derived chemokine) support trends observed on RayBiotech G series 3 cytokine Ab arrays. Cytokine levels were determined using the RayBiotech Quantibody service.

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5 The online version of this article contains supplemental material.
FIGURE 4. Nlrp3- and Asc-deficient mice demonstrated significantly increased mortality following K. pneumoniae airway infection. Mice were intratracheally challenged with (7.4×10⁴) CFU of K. pneumoniae and survival was assessed over the course of 4 days. A, Total RNA was extracted from whole, homogenized lungs and expression was determined via real time PCR. NLRP3 expression was dramatically increased 48 h post-K. pneumoniae infection. B and C, Mice lacking Nlrp3 (B) or Asc (C) demonstrated significantly increased mortality compared with WT mice (p < 0.05; log rank test). D, No significant difference in survival was observed between WT mice and animals lacking Nlrc4 following K. pneumoniae challenge. Mock, n = 7; Nlrp3−/−, n = 11; Asc−/−, n = 4; Nlrc4−/−, n = 7; WT, n = 19.

To determine whether inflammation was also reduced in vivo, Asc- and Nlrp3-deficient mice were challenged with (7.4×10⁴) CFU of K. pneumoniae delivered through airway infection. Previous reports show that E. coli LPS enhances NLRP3 expression in vitro (36). To assess this issue in animals in the context of K. pneumoniae, we show that in vivo NLRP3 expression is greatly induced (600 times) by these bacteria while ASC expression is increased 25 times (Fig. 4A). To determine whether NLRP3 and ASC are involved in mediating the overall host response to K. pneumoniae in vivo, animals were challenged via intratracheal instillation and survival was assessed over the course of 4 days. Mice lacking the Nlrp3 gene demonstrated moderate but statistically significant increased mortality compared with WT mice (p < 0.05; log rank test) (Fig. 4B). Asc-deficient mice demonstrated similar increases in mortality (Fig. 4C). No significant difference in survival was observed between Nlrc4-deficient mice and the WT controls (Fig. 4D). This moderate effect of Nlrp3 on animal survival may be explained by other NLRs that can mediate inflammasome formation in the lung, as well as by the compensatory increase in other inflammatory cytokines, such as IL-6, which might mask the effect of Nlrp3 deficiency in animals.

Nlrp3-deficient mice demonstrate significantly attenuated airway inflammation following K. pneumoniae infection

The above results suggested that NLRP3 is an important mediator of several inflammatory cytokines and is essential for mouse survival in an in vivo model of K. pneumoniae infection. To determine whether airway inflammation was attenuated, Nlrp3-deficient mice were challenged via intratracheal instillation with K. pneumoniae and the lungs were harvested 48 h postinoculation for histology analysis. Lung sections were prepared to reveal the main bronchi of the left lobe from the indicated genotypes, and representative sections were examined (×10 and ×20 original magnification) (Fig. 5A). In comparison to WT and Nlrc4−/− mice, Nlrp3−/− mice show decreased inflammatory cell recruitment and less occlusion of the alveolar spaces following K. pneumoniae infection. Mice were challenged with 7.4×10⁴ CFU of K. pneumoniae. Whole lungs were harvested 48 h postinfection, and each lobe was assessed and scored at specific locations along the main bronchi. Representative histology sections from the apical region of the main bronchi of the large lobe (×10 original magnification) are shown. B–E, Histology images were evaluated for a variety of inflammatory parameters and scored between 0 (absent) and 3 (severe). Significant attenuation in airway inflammation was observed in K. pneumoniae-challenged mice lacking Nlrp3 (p < 0.05). Mock, n = 3; Nlrp3−/−, n = 6; Nlrc4−/−, n = 6; WT, n = 12.

In vivo levels of IL-1β and cell death are reduced in K. pneumonia-challenged Nlrp3−/− mice

We have demonstrated that NLRP3 is required for necrosis in both human and mouse cell cultures challenged with Klebsiella. We further demonstrate that NLRP3 mediates pulmonary inflammation and protects against bacterial infection. We next sought to assess the in vivo relevance of this gene in cytokine production,
bacteria-induced necrosis, and HMGB1 release. Processes that regulate the latter two have not been extensively studied in an in vivo setting. To determine the physiologic effects of NLRP3, we first measured IL-1β levels in the BALF and serum of WT and NLRP3−/− mice. Deletion of Nlrp3 caused a near abrogation of serum IL-1β, which is consistent with the important role NLRP3 plays in IL-1β production by monocytes (Fig. 6A). Deletion of Asc also resulted in marked decreases in the circulating levels of IL-1β (Fig. 6A). IL-1β in the BALF was modestly reduced in the Nlrp3−/− mice, a finding that is similar to that of other

**FIGURE 6.** NLRP3-regulated *Klebsiella pneumoniae* induced IL-1β and necrotic cell death in vivo. *A.* Serum levels of IL-1β were significantly reduced in both Nlrp3−/− and Asc−/− mice challenged with *K. pneumoniae* (*p < 0.05*). *B,* A modest yet significant decrease in *K. pneumoniae*-induced IL-1β was observed in the BALF of Nlrp3−/− mice compared with that of WT mice (*p < 0.05*). *C* and *D,* In contrast, IL-6 levels were elevated in the serum (*C*) and BALF (*D*) of Nlrp3−/− mice following *K pneumoniae* infection. IL-1β and IL-6 were measured by ELISA. *E,* Decreased levels of cell death were detected in the BALF of *K pneumoniae*-infected, Nlrp3−/− mice as determined by LDH release. *F,* Serum levels of HMGB1 were dramatically reduced in mice lacking Nlrp3 as determined by Western blot analysis. *G,* Cells obtained by BALF were collected from *K. pneumoniae*-challenged WT and Nlrp3−/− animals and were subjected to electron microscopy analysis. Cells from WT mice exhibited cell death features morphologically consistent with necrosis. In contrast, cells obtained from Nlrp3−/−deficient animals did not exhibit a morphology indicative of cell death. Magnified intracellular *K. pneumoniae* are shown in the two top panels and in the bottom panel at the far right.

in vivo analyses (13). The modest effect is likely due to redundant NLR proteins in the lung stroma that remained intact in these mice. This decrease in IL-1β was accompanied by a modest increase of IL-6 observed between Nlrp3−/− and WT mice in serum and BALF samples (Fig. 6, *C* and *D*), which is consistent with the cytokine array analysis of in vitro cultured cells shown in Fig. 3. No significant differences were observed in the cellular composition of the BALF between genotypes (supplemental Table S2). Deletion of Nlrp3 also decreased overall levels of cell death in BALF samples as determined by LDH release (Fig. 6E). To examine whether NLRP3 is responsible for the induction of HMGB1, HMGB1 levels were measured in serum samples of *K. pneumoniae*-challenged mice. As measured by Western blot analysis, *Klebsiella*-induced HMGB1 release was substantially abrogated in Nlrp3−/− mice (Fig. 6F). Having determined that NLRP3 was involved in the initiation of cell death in response to *K. pneumoniae* in vivo, we sought to determine whether this cell death was morphologically consistent with necrosis. Cells collected from BALF samples obtained from *K. pneumoniae*-infected animals were subjected to electron microscopy analysis. Cells harvested from WT animals demonstrate several morphological features consistent with necrosis, including loss of plasma membrane integrity and lack of chromatin condensation (Fig. 6G). In contrast, cells obtained from Nlrp3−/−deficient animals exhibit no signs of cell death, despite the presence of several intracellular *K pneumoniae* bacteria (Fig. 6G). These results indicate that Nlrp3 mediates *K pneumoniae*-induced inflammation and cell death in vivo and that this cell death is morphologically and biochemically consistent with necrosis.

**Discussion**

Several recent reports have established a role for NLRP3 in mediating pathogen-induced inflammation in vitro, but the majority of evidence has been provided using ex vivo cultures obtained from gene-deleted mice. In this study, we identify NLRP3 as a critical effector of the host immune response to *K. pneumoniae*, a major cause of community-acquired bacterial pneumonia. *K. pneumoniae* was selected for analysis because it induces a flagrant cytokine and inflammatory response, including the release of HMGB1, and significant necrosis in the lung, thus allowing us to assess the effect of NLRP3 and ASC on all three processes. This is the first combined in vitro and in vivo demonstration that an NLR molecule is a key regulator of not only IL-1β maturation but also HMGB1 release and necrotic cell death in response to pathogen exposure. Furthermore, these results demonstrate the in vivo consequences of NLRP3 activity on host survival and inflammation. Despite substantial decreases in lung inflammation and tissue destruction (as evaluated by cytokine analysis and criteria specified in Materials and Methods), mice lacking Nlrp3 demonstrate increased susceptibility to *Klebsiella*-induced lethality. This finding confirms that NLRP3 activity contributes to protective host responses to bacterial pathogens via both inflammasome-dependent and independent processes. This is similar to recent findings regarding the role of NLRP3 during infection by influenza virus, where the Nlrp3 gene is found to be important for increased pulmonary inflammation, cellular infiltrate, and IL-1β/IL-18 production, all of which coincide with increased animal survival and decreased viral load (37, 38).

After *S. flexneri*, *K. pneumoniae* is the second Gram-negative bacterial pathogen identified that activates the NLRP3-dependent cell death program termed pyroptosis (8). This pathway of cell death has morphological features characteristic of necrosis and,
similar to necrosis, is inherently proinflammatory. Cellular components spill out from the pyronecrotic cell into the microenvironment. Among these components is HMGB1, a nuclear protein that takes on the role of a powerful proinflammatory cytokine when released from the cell (17, 39). HMGB1 stimulates the RAGE, TLR2, and TLR4 receptors on neighboring monocytes and macrophages and results in the induction of several inflammatory cytokines, including TNF-α and IL-1β (40–42). K. pneumoniae induces a significant increase in the systemic levels of HMGB1 in the WT mice, whereas no HMGB1 is observed in the serum from Nlrp3−/− animals. This NLRP3-dependent release of HMGB1 is also observed in human THP-1 monocytic cells challenged with Klebsiella. Caspase-1 inhibitors failed to abrogate NLRP3-mediated HMGB1 release, suggesting that this phenomenon does not require inflammasome activity. It should be noted that HMGB1 levels are significantly increased in human septic patients, including those with K. pneumoniae sepsis (27). Neutralization of HMGB1 is currently under investigation as a therapeutic target for the intervention of sepsis, bacteremia, and induced acute respiratory distress syndrome (43, 44). Although a broad inhibition of NLRP3-dependent responses may be detrimental to host survival, neutralization of HMGB1 may provide an opportunity to abrogate NLRP3-mediated inflammation without increasing host mortality.

Pyronecrosis serves as an interesting contrast to pyroptosis, another form of pathogen-induced cell death (45). Although pyronecrosis requires caspase B but not caspase activity, pyroptosis requires the activity of caspase-1 (21). These two pathways also appear to be induced by different stimuli and involve different NLRs. Pyronecrosis has been observed in monocytic cells infected with 50 MOI of Shigella flexneri, an MOI previously shown to cause necrosis (8). Pyroptosis has been observed in monocytic cells infected with Salmonella (31, 46), Pseudomonas aeruginosa, and low-dose (≤10 MOI) S. flexneri (31, 46–48). Interestingly, although ASC is required for the activation of caspase-1, the deletion of ASC does not abrogate caspase-1-dependent pyroptosis initiated by P. aeruginosa (47).

Our in vitro and in vivo results indicate that NLRP3-dependent pyronecrosis is the predominant cell death and inflammation pathway induced by Klebsiella. In contrast to the Salmonella-induced pyroptosis pathway, which is dependent on caspase-1 and NLRC4, ablation of Nlrc4 or the caspase-1 gene has minimal effect on inflammation or cell death induced by K. pneumoniae. Interestingly, caspase B inhibitors not only block NLRP3-mediated pathogen induced cell death but also block the induction of IL-1β maturation by K. pneumoniae. A similar requirement for caspase B in NLRP3-mediated IL-1β maturation in response to silica crystals, aluminum salts, and lysosomal permeabilization has also been recently reported (16, 49). These findings suggest that a significant portion of K. pneumoniae-induced IL-1β release might lie downstream of pyronecrosis, which is not surprising given the strong proinflammatory activity of HMGB1.

Previously, we demonstrated that NLRP3 and ASC were required for the release of IL-1β and HMGB1 from a human macrophage cell line THP-1 infected with S. flexneri. In the current study we have shown that the release of IL-1β and HMGB1 is also markedly decreased from Nlpr3− and Asc-deficient macrophages infected with K. pneumoniae and have extended this finding to a study of the whole animal. Furthermore, our results reveal additional cytokines and chemokines, such as GM-CSF and IL-1α, which are also reduced in the absence of Nlpr3 or Asc. Perhaps to compensate for the lack of these inflammatory mediators, the levels of MIP-1α, IL-6, and RANTES were increased in Nlpr3−/− or Asc−/− cells. The mechanisms by which NLRP3 and ASC regulate the production of these cytokines and chemokines are currently under investigation; however, these results demonstrate that NLRP3 and ASC may directly or indirectly influence the inflammatory response to K. pneumoniae through both inflammasomedependent and -independent pathways.

In summary, our results indicate that NLRP3 and ASC are key regulators of necrosis and inflammation associated with K. pneumoniae infections in human and mouse cells. Most importantly, this body of work demonstrates that altering specific NLRs can dramatically affect the in vivo host immune response in a common and clinically relevant model of Gram-negative bacterial pneumonia. Given the clear phenotype observed in the Nlpr3− and Asc-deficient mice, it will be interesting to further define the mechanisms underlying the attenuation of inflammation and increased mortality, with special emphasis on the role of HMGB1. Our findings suggest the cumulative effect of NLRP3 on cytokines and chemokines, cell death, and HMGB1 protects the host from pathogen-induced mortality. These findings exemplify the complex interactions associated with NLRP3 in innate immune signaling and the potential limitations of therapeutic intervention in NLRP3 activity.

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Disclosures

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